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Extraction, Chemical Composition and *in vitro* Antibacterial Activity of the Essential Oil of *Lippia origanoides* Kunth from Northeast Brazil

Monique Ellen Torres da Silva[1,2*]
https://orcid.org/0000-0002-4540-475X

Antônia Lucivânia de Sousa Monte[2]
https://orcid.org/0000-0003-2504-5136

Marlene Nunes Damaceno[2]
https://orcid.org/0000-0002-3553-6740

Renata Chastinet Braga[2]
https://orcid.org/0000-0001-6630-2835

Jane Sélia dos Reis Coimbra[3]
https://orcid.org/0000-0002-5998-189X

Srinivasan Ramanathan[4]
https://orcid.org/0000-0003-2697-3137

Felipe Alves de Almeida[5]
https://orcid.org/0000-0002-8020-6601

[1]Universidade Federal de Juiz de Fora, Departamento de Farmácia, Governador Valadares, Minas Gerais, Brasil; [2]Instituto Federal de Educação, Ciência e Tecnologia do Ceará, Departamento de Tecnologia de Alimentos, Limoeiro do Norte, Ceará, Brasil; [3]Universidade Federal de Viçosa, Departamento de Tecnologia de Alimentos, Viçosa, Minas Gerais, Brasil; [4]Fujian Agriculture and Forestry University, College of Life Sciences, Fuzhou, Fujian, China; [5]Universidade Federal de Juiz de Fora, Departamento de Nutrição, Governador Valadares, Minas Gerais, Brasil.

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*Correspondence: monique.ellen@ufjf.edu.br (M.E.T.S).

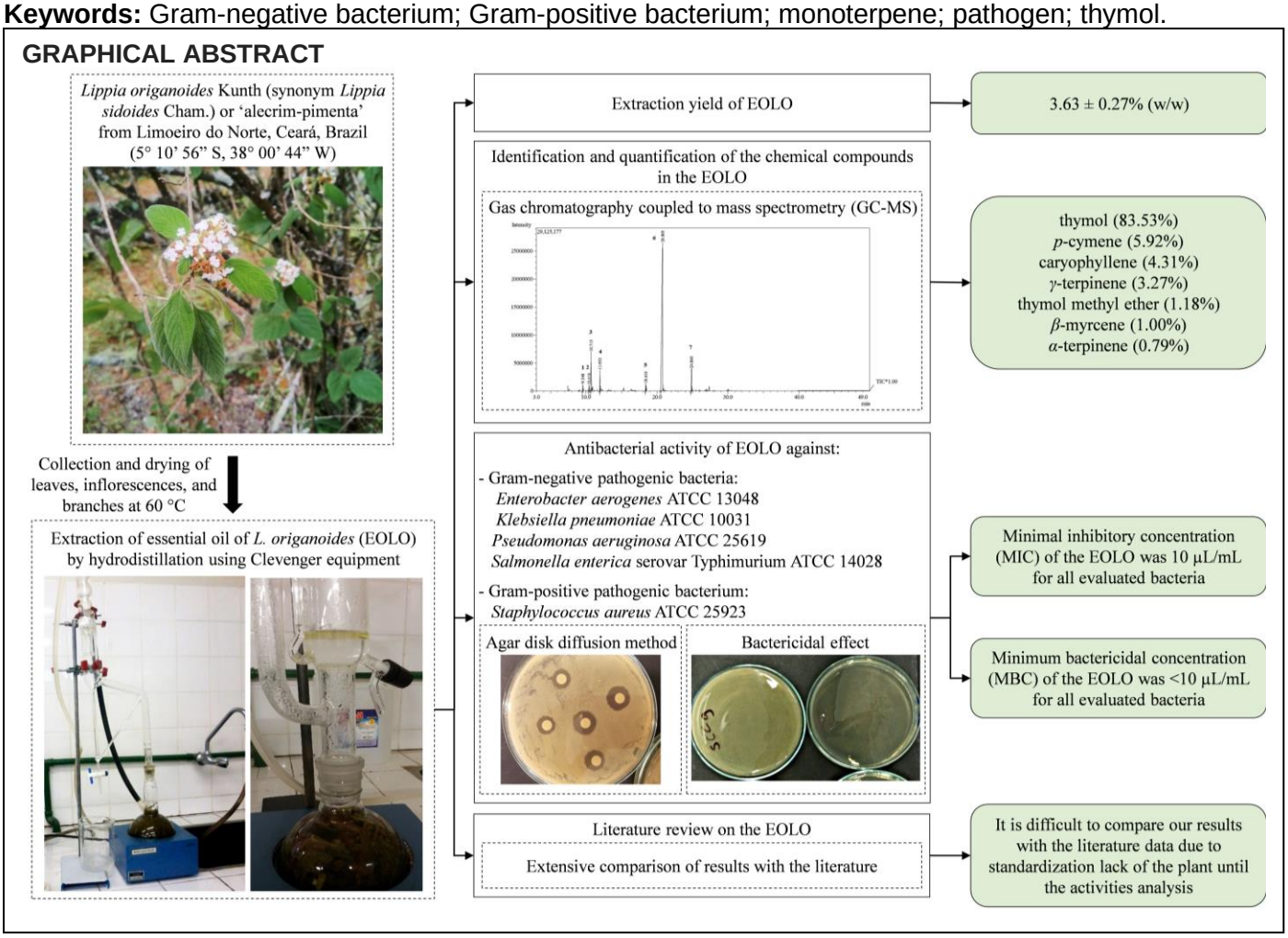
HIGHLIGHTS

- Thymol was identified as the main metabolite in the EOLO.
- EOLO has antibacterial activity against Gram-negative and Gram-positive bacterial pathogens.
- The antibacterial effect of EOLO may be associated with the presence of thymol.

Abstract: *Lippia origanoides* Kunth has among its synonyms *Lippia sidoides* Cham. and is popularly known as 'alecrim-pimenta' or 'rosemary pepper'. Essential oil of *L. origanoides* (EOLO) has been previously reported for several biological activities without significant acute toxicity, even at higher doses. In this study, essential oil (EO) was extracted from dried leaves, inflorescences, and branches of *L. origanoides* from Limoeiro do Norte, Ceará, Brazil by hydrodistillation at 3.63% (w/w) yield. Posteriorly, the presence of chemical compounds in the EOLO was identified by gas chromatography coupled to mass spectrometry (GC-MS). The analysis allowed to identify the thymol as the main metabolite (83.53%), followed by *p*-cymene (5.92%), caryophyllene (4.31%), *γ*-terpinene (3.27%), thymol methyl ether (1.18%), *β*-myrcene (1.00%), and *α*-terpinene (0.79%). The minimal inhibitory concentration (MIC) of EOLO was assessed against Gram-negative and Gram-positive bacterial pathogens such as *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella enterica* serovar Typhimurium, and *Staphylococcus aureus*. At 10 µL/mL concentration, the EOLO exhibited antibacterial activity against all the tested bacterial pathogens, and this concentration was considered as the MIC. The minimum bactericidal concentration (MBC) of EOLO was assessed at the MIC concentration, which inhibited the complete growth of all tested bacterial pathogens after 3 h. Therefore, concentrations less than 10 µL/mL were set as MBC. Thus, our findings suggest that the EOLO can be used as a promising antibacterial agent in the industries.

Keywords: Gram-negative bacterium; Gram-positive bacterium; monoterpene; pathogen; thymol.

GRAPHICAL ABSTRACT



INTRODUCTION

Essential oils (EO) are volatile organic constituents responsible for the fragrance in several plant species. Many research groups documented the importance of these compounds in the food, pharmaceutical, and agricultural fields. Indeed, EO are known to potent several pharmacological activities such as antimicrobial, anti-inflammatory, antipyretic, gastroprotective, and antioxidant properties. Besides, these EO have been used as phytosanitary controls since they allow the development of techniques to reduce the adverse effects of oxidants, radicals, pathogenic and spoilage microorganisms [1-6].

Among the several genera of EO-producing plants, the *Lippia* stands out. The genus *Lippia* (Verbenaceae) comprises approximately 200 species of herbs, shrubs, and small trees, distributed in Central and South America, as well as in Tropical Africa. It is estimated that Brazil contains 70-75% of all known species and a large part is concentrated in the Northeast region [7-9]. *Lippia origanoides* Kunth has among its synonyms *Lippia sidoides* Cham. and is popularly known as 'alecrim-pimenta' or 'rosemary pepper'. This is an aromatic plant found naturally in Northeast Brazil and used in popular medicine as an antiseptic, scabicide, and anti-inflammatory [10,11]. The EO of *L. origanoides* (EOLO) has been reported for several biological activities [8-75], such as acaricidal [8,32], allelopathic [42], anesthetic [9,72], anthelmintic [20,21], antibacterial [12,17,23,24,34,38-41,48,51,70,71,73,74], antidepressant [56], antifungal [14,22,26,28,29,31,33,50,52,62], antigingivitis [57,60], anti-inflammatory [18,45,75], antimicrobial [18,19,43,47,58], antioxidant [24,45,69], antiparasitic [35,68], antiplaque [57,60], antiprotozoal [27,44,49,53,61,63], cytotoxicity [29,49,61,63], gastroprotective [45], insecticidal [13,30,36,37,54,55,62,65,66], larvicidal [10,25], phytotoxicity [29,50], sciatic nerve excitability [16], and toxicity [31,35,37,45,49,68]. Interestingly, to the best of our knowledge, EOLO has not been previously reported for any acute toxic effect even at high doses above 500 mg/kg [12].

EOLO is rich in terpenes, mainly carvacrol [8,9,13,27,33,34,36,58,63,64] and thymol [10,14-17,19,21,22,24-26,28-32,35,37,38,40-42,45,49,50,52-55,57,59,61,66-75], responsible for their antibacterial and antifungal activities [76,77]. However, it is very known that the activity against bacteria by a range of EO is dependent on the mechanisms of action of each individual compound or the combinations between them, such as synergistic, additive, and antagonistic interactions [76,78,79]. Thus, this combination of compounds

with different mechanisms of action can significantly increase the antibacterial efficacy of EO against multidrug-resistant bacterial pathogens, and which also reducing the emergence of novel resistant species [73,76,80].

Bacterial resistance to antibiotics is increasing, so there is a growing concern in opportunistic bacterial pathogens that cause healthcare-associated infections (HAIs), such as *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. Foodborne pathogens are also a concern, particularly *Salmonella enterica* serovar Typhimurium [81]. The use of EO to pathogenic bacteria control should begin with the *in vitro* antibacterial activity study. For this, some factors must be considered and evaluated together, such as i) EO extraction yield, an important aspect from an economic point of view; ii) chemical composition of EO, which affects the biological activity; iii) EO concentration and iv) the contact time required to have bactericidal and bacteriostatic activities, which influence its applicability [11,82,83].

Therefore, the present study aimed to extract the EOLO from Northeast Brazil and evaluate its chemical composition by gas chromatography coupled to mass spectrometry (GC-MS). Further, *in vitro* antibacterial activity against the bacterial pathogens *E. aerogenes*, *K. pneumoniae*, *P. aeruginosa*, *Salmonella* Typhimurium, and *S. aureus* was evaluated.

MATERIAL AND METHODS

Plant material

On February 03, 2014, plant of *L. origanoides* Kunth from the Unidade de Estudo, Pesquisa e Extensão (UEPE) of the Instituto Federal do Ceará (IFCE), Limoeiro do Norte, Ceará, Brazil (5° 10’ 56” S, 38° 00’ 44” W) was compared with the plant of *L. sidoides* Cham. collected in 2005 at the Universidade Federal de Sergipe (UFS) *campus* rural, São Cristóvão, Sergipe, Brazil (10º 33’ 19” S, 37º 3’ 43” W) and deposited in Reflora – Herbário Virtual (barcode ASE0018058; http://reflora.jbrj.gov.br/reflora/herbarioVirtual/ConsultaPublicoHVUC/ConsultaPublicoHVUC.do). It is worth mentioning that the accepted scientific name is *L. origanoides* Kunth and *L. sidoides* Cham. is one of its synonyms according to Brazilian Flora 2020 [84]. Posteriorly, the leaves, inflorescences, and branches of *L. origanoides* were collected at 9 am on February 10, 2014, washed, disinfected with sodium hypochlorite (NaClO) at 1% (w/v) for 1 min, dried in a circulating air oven at 60 ºC for 6 h, and immediately the extraction of the EO was carried out.

Extraction of essential oil of *L. origanoides* (EOLO)

The EO was extracted from 200 g of dried leaves, inflorescences, and branches of *L. origanoides* by hydrodistillation using Clevenger equipment (Glasslabor, Brazil) [85], which consists of the evaporation of a mixture of water and volatile compounds present in the plant material as described in the European Pharmacopoeia [86]. The extraction was conducted for 90 min at 100 ºC in a heating blanket. Then, the water was removed by decanting, and the obtained EOLO was stored at 4 ºC in the refrigerator, protected from light. This extraction was performed 8 times, totaling 1.6 kg of dry plant material. Further, the obtained yield was measured at each extraction and expressed in % (w/w).

Identification and quantification of the chemical compounds in the EOLO

The identification and quantification of the chemical compounds in the EOLO were performed by GC-MS. The GC-MS model QP2010 (Shimadzu, Japan), with an automatic injector AOC-20i and capillary column RTX-5MS (5% phenyl and 95% dimethylpolysiloxane, 30 m x 0.25 mm x 0.25 µm) was used. The flow of helium gas through the capillary column was adjusted to 1.0 mL/min. The oven temperature was set to 40 to 180 ºC at a rate of 4 ºC/min, then 180 to 280 ºC at a rate of 20 ºC/min, and then kept at 280 ºC for 10 min. The temperatures in the injector and detector were kept at 250 and 300 ºC, respectively. The mass spectra of the isolated compounds were collected by GC-MS with an electronic impact of 70 eV. The compounds were identified by comparing their mass spectra with those of the NIST08 library and published data [87]. The compound concentrations were determined from the peak areas on the chromatogram.

Antibacterial activity of EOLO

Bacterial strains

The evaluation of the antibacterial potential of EOLO was performed for the Gram-negative bacterial pathogens *E. aerogenes* ATCC 13048, *K. pneumoniae* ATCC 10031, *P. aeruginosa* ATCC 25619, and *Salmonella* Typhimurium ATCC 14028, and the Gram-positive bacterial pathogen *S. aureus* ATCC 25923.

All isolates were obtained by the culture collection of the Laboratory of Microbiology at the Universidade Federal do Ceará (UFC; Ceará, Brazil). The bacterial pathogens were stored at -80 °C in Brain Heart Infusion broth (BHI broth; Himedia, India) with 20% (v/v) sterilized glycerol.

Preparation of the standardized inoculum

Before all experiments, the 5% (v/v) of bacterial strains from glycerol stock were cultivated in BHI broth at 37 ºC. After 24 h of incubation, the broths were plated in BHI agar (Merck, Germany) and incubated at 37 ºC for 18 h. Subsequently, the isolated colonies were inoculated directly into the BHI broth, and their turbidity was adjusted to 0.5 using a McFarland scale, corresponding to a standardized inoculum with a concentration of approximately 1.5 x 10^8 colony-forming units per milliliter (CFU/mL) [88].

Agar disk diffusion method

The agar disk diffusion assay was performed for assessing the antibacterial activity of EOLO according to the methods previously described by Oliveira and coauthors [51] and Cavalcanti and coauthors [8], with some modifications. Briefly, the standardized inoculum of each bacterium was uniformly distributed on a Petri dish containing solidified Mueller-Hinton agar (MH agar; Merck, Germany) using a sterile swab. Then, the sterile white paper discs with 6 mm in diameter (Whatman no. 5) were soaked with 20 µL of solutions at 0.0, 1.25, 2.5, 5.0, 10.0, 20.0, 40.0, 80.0, and 160.0 µL/mL of the EOLO. After absorbing all of the EOLO, the discs were placed on the MH agar containing the bacterial inoculum and incubated at 37 ºC. After 24 h, the diameter of the inhibition zones were measured and expressed in mm. The minimal inhibitory concentration (MIC) was considered to be one in which the diameter of the zone of inhibition was greater than or equal to 10 mm ($\geq$ 10 mm) [89]. This experiment was performed in five biological replicates. The values of the replicates were used for the analysis of variance followed by Tukey's test using the Statistical Analysis System and Genetics Software (Sisvar) and a p-value less than 0.05 was statistically significant.

Bactericidal effect

The bactericidal effect of the MIC of EOLO was evaluated according to the methods of Sforcin and coauthors [90] and Oliveira and coauthors [51], with some modifications. The MIC of EOLO considered for all evaluated bacteria was 10 µL/mL, corresponding to 1% (v/v). Thus, a volume of 3 mL of a solution containing 10 µL/mL of EOLO, 0.2% (v/v) of Tween 80 (Merck, Brazil), and standardized inoculum of each bacterium were prepared and incubated at 37 °C. After 0, 3, 6, 9, 21, and 24 h of incubation, aliquots of 0.1 mL of this solution were plated by spread plate method on MH agar with a Drigalski spatula and incubated at 37 ºC for 24 h. Subsequently, the colonies were counted and the number of logarithm base 10 of the number of CFU/mL (log cycles CFU/mL) was calculated for each bacterium. The negative control was performed replacing the EOLO with sterile distilled water. This experiment was performed in five biological replicates.

RESULTS

Extraction and chemical composition of EOLO

The extraction yield of EOLO from plant materials dried in a circulating air oven at 60 ºC for 6 h was 3.63 ± 0.27% (w/w), and seven chemical compounds were identified in the EOLO (Figure 1 and Figure 2).

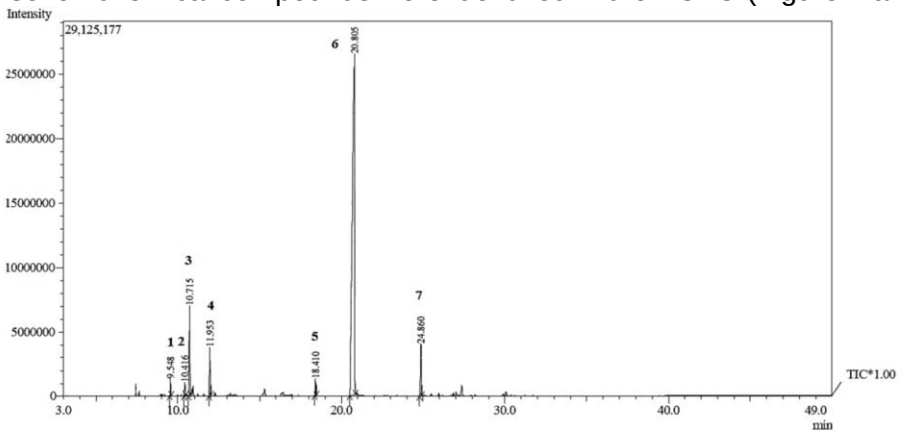
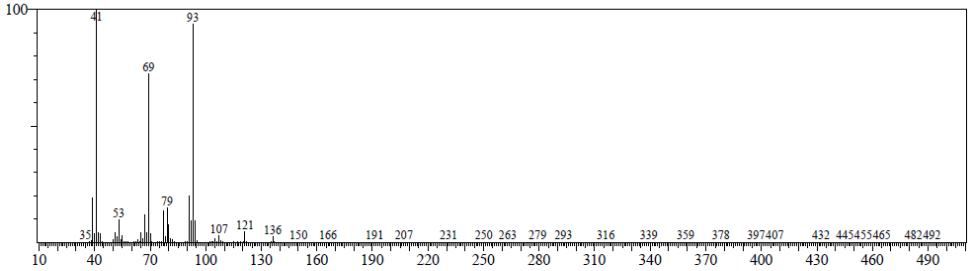


Figure 1. Chromatogram of the essential oil of *Lippia origanoides* Kunth (EOLO) obtained by gas chromatography (GC) with peaks identification by mass spectrometry (MS): peak 1 – *β*-myrcene; peak 2 - *α*-terpinene; peak 3 - *ρ*-cymene; peak 4 - *γ*-terpinene; peak 5 - thymol methyl ether; peak 6 – thymol; peak 7 – caryophyllene. The relative percentage of each compound is shown in Table 2.

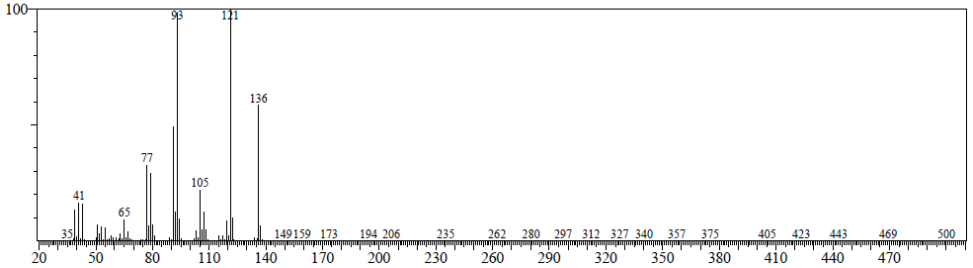
Peak 1

Line#:1 R.Time:9.550(Scan#:1311) MassPeaks:263 BasePeak:41.10(286677)
RawMode:Averaged 9.545-9.555(1310-1312) BG Mode:Calc. from Peak



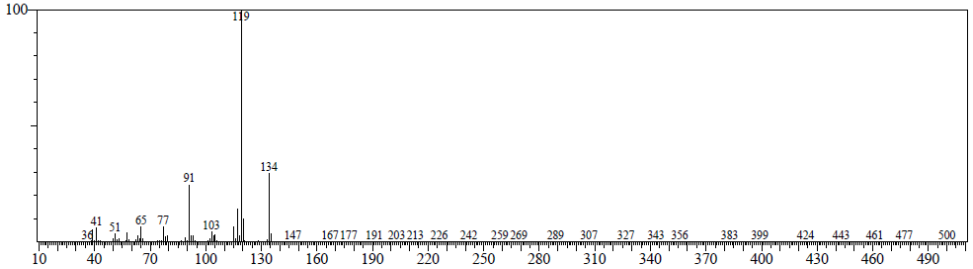
Peak 2

Line#:2 R.Time:10.415(Scan#:1484) MassPeaks:277 BasePeak:121.15(163888)
RawMode:Averaged 10.410-10.420(1483-1485) BG Mode:Calc. from Peak



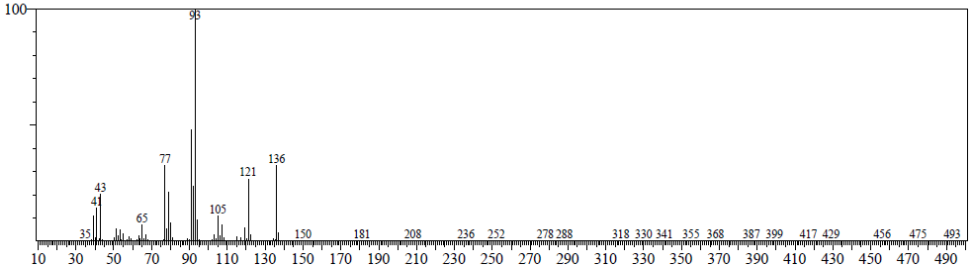
Peak 3

Line#:3 R.Time:10.715(Scan#:1544) MassPeaks:264 BasePeak:119.15(2591289)
RawMode:Averaged 10.710-10.720(1543-1545) BG Mode:Calc. from Peak



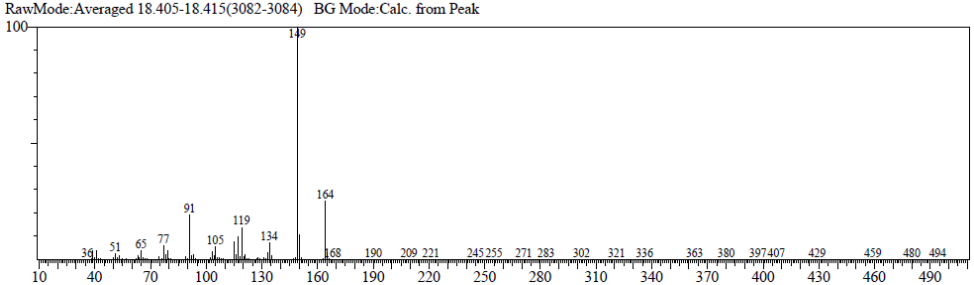
Peak 4

Line#:4 R.Time:11.955(Scan#:1792) MassPeaks:294 BasePeak:93.10(821950)
RawMode:Averaged 11.950-11.960(1791-1793) BG Mode:Calc. from Peak



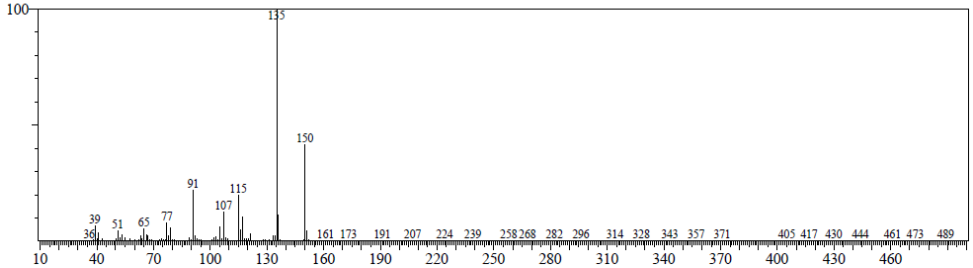
Peak 5

Line#:5 R.Time:18.410(Scan#:3083) MassPeaks:315 BasePeak:149.15(460171)
RawMode:Averaged 18.405-18.415(3082-3084) BG Mode:Calc. from Peak



Peak 6

Line#:6 R.Time:20.805(Scan#:3562) MassPeaks:319 BasePeak:135.15(8169275)
RawMode:Averaged 20.800-20.810(3561-3563) BG Mode:Calc. from Peak



Peak 7

Line#:7 R.Time:24.860(Scan#:4373) MassPeaks:307 BasePeak:93.10(277782)
RawMode:Averaged 24.855-24.865(4372-4374) BG Mode:Calc. from Peak

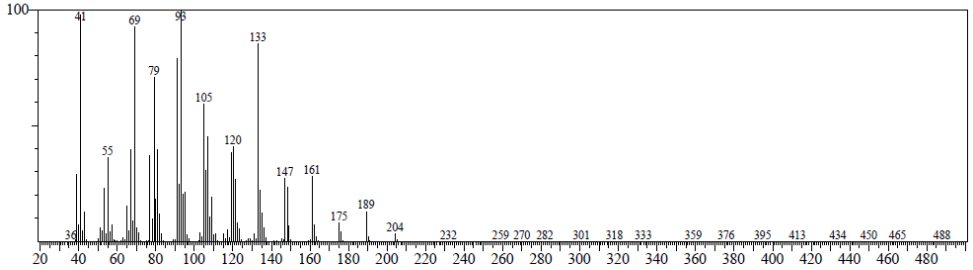


Figure 2. Mass spectrum of the peaks obtained by gas chromatography (GC) of the essential oil of *Lippia origanoides* Kunth (EOLO): peak 1 – *β*-myrcene; peak 2 - *α*-terpinene; peak 3 - *ρ*-cymene; peak 4 - *γ*-terpinene; peak 5 - thymol methyl ether; peak 6 – thymol; peak 7 – caryophyllene.

The identified compounds were considered as 100% of the composition of the EOLO to calculate the percentage of each compound, as shown in Table 1. The thymol (83.53%) was the main metabolite identified in the EOLO, followed by *p*-cymene (5.92%), caryophyllene (4.31%), *γ*-terpinene (3.27%), thymol methyl ether (1.18%), *β*-myrcene (1.00%), and *α*-terpinene (0.79%). Thus, the aromatic monoterpenes represent 90.63% of the EOLO (Table 1).

Table 1. Chemical compounds identified and quantified in the essential oil of *Lippia origanoides* Kunth (EOLO) by gas chromatography coupled to mass spectrometry (GC-MS).

Peak*	RT (min)	[M-H] (*m/z*)	Percentage of similarity (%)	Chemical compound	Relative percentage (%)	Classification
6	20.805	135.15	95	thymol	83.53	Aromatic monoterpene
3	10.715	119.15	96	*p*-cymene	5.92	Aromatic monoterpene
7	24.860	93.10	96	caryophyllene	4.31	Sesquiterpene
4	11.953	93.10	97	*γ*-terpinene	3.27	Monoterpene
5	18.410	149.15	97	thymol methyl	1.18	Aromatic monoterpene
1	9.548	41.10	96	*β*-myrcene	1.00	Acyclic monoterpene
2	10.416	121.15	94	*α*-terpinene	0.79	Monoterpene
				TOTAL	**100.00**	

*Peaks identified in the chromatogram in Figure 1; RT = Retention time; [M-H] = Molecular ion.

Antibacterial activity of EOLO

The EOLO showed efficient antibacterial activity against the Gram-negative bacterial pathogens *E. aerogenes*, *K. pneumoniae*, *P. aeruginosa*, and *Salmonella* Typhimurium, and the Gram-positive bacterial pathogen *S. aureus* (Table 2). The most significant antibacterial activity of EOLO was observed against *S. aureus* with an inhibition zone of 26.6 mm at 160 µL/mL. Moreover, the antibacterial activity of EOLO was depended on the concentration for all tested bacterial pathogens (Table 2).

Table 2. Antibacterial activity of essential oil of *Lippia origanoides* Kunth (EOLO) by agar disk diffusion method.

Concentration of EOLO (µL/mL)	Mean of the diameter of the inhibition zone ± standard deviation (mm)				
	E. aerogenes	*K. pneumoniae*	*P. aeruginosa*	*Salmonella* Typhimurium	*S. aureus*
0.00	0.0 ± 0.0^{e}	0.0 ± 0.0^{f}	0.0 ± 0.0^{f}	0.0 ± 0.0^{f}	0.0 ± 0.0^{e}
1.25	0.0 ± 0.0^{e}	0.0 ± 0.0^{f}	0.0 ± 0.0^{f}	0.0 ± 0.0^{f}	0.0 ± 0.0^{e}
2.50	7.2 ± 0.8^{d}	6.7 ± 1.0^{e}	8.2 ± 0.8^{e}	7.9 ± 0.8^{e}	7.3 ± 0.3^{d}
5.00	8.5 ± 0.5^{d}	9.1 ± 0.5^{d}	9.7 ± 0.3^{de}	8.2 ± 0.8^{e}	8.6 ± 0.4^{cd}
10.00	**11.7 ± 0.3**^{c}	**10.6 ± 0.4**^{d}	**10.3 ± 0.7**^{d}	**11.8 ± 0.5**^{de}	**10.2 ± 0.8**^{cd}
20.00	13.0 ± 0.5^{bc}	14.2 ± 0.5^{c}	14.1 ± 0.2^{c}	14.3 ± 1.2^{cd}	15.4 ± 0.4^{bc}
40.00	14.5 ± 0.7^{b}	16.3 ± 0.5^{b}	15.3 ± 0.6^{bc}	16.4 ± 1.1^{bc}	18.4 ± 1.1^{b}
80.00	15.0 ± 1.0^{b}	17.0 ± 2.1^{b}	16.0 ± 0.7^{b}	19.8 ± 2.5^{b}	22.2 ± 2.6^{ab}
160.00	20.6 ± 1.5^{a}	19.4 ± 0.5^{a}	21.4 ± 1.7^{a}	24.8 ± 2.9^{a}	26.6 ± 6.2^{a}

The minimal inhibitory concentration (MIC) was considered to be one in which the diameter of the inhibition zone was greater than or equal to 10 mm (≥ 10 mm) [89]; In bold = MIC of the EOLO; Means followed by different superscript letters in the same column differ among themselves at 5% probability ($p < 0.05$) by Tukey's test.

In this study, the classification of the diameter of the zones of inhibition was done by the method of Carović-Stanko and coauthors [89]. According to this method, the inhibitory activity of substances must be at least 10 mm in diameter of zones of inhibition. These authors also classified the inhibitory activity as moderate (10 to 15 mm) or strong (> 15 mm) based on the zones of inhibition. Thus, the concentration of 10 µL/mL of the EOLO was considered the MIC for all the tested bacterial pathogens. Further, we observed that the inhibition zones ranged from 10.2 to 11.8 mm at this MIC concentration (Table 2). Subsequently, the bactericidal effect at the MIC concentration was evaluated in solution for 24 h. The obtained data revealed that the MIC of EOLO completely inhibited the growth of all bacterial pathogens tested after 3 h, in contrast to their respective untreated controls. Moreover, the MIC of EOLO did not allow the bacterial suspension to resume its growth up to the tested time interval of 24 h (Figure 3). Interestingly, EOLO at 10 µL/mL required only a minimum contact time of less than 0 h, at this time the EO was able to inhibit more than 2 log cycles of CFU/mL (Figure 3). The result of this assay has revealed that less than 10 µL/mL of EOLO was considered as minimum bactericidal concentration (MBC) for all bacterial pathogens tested.

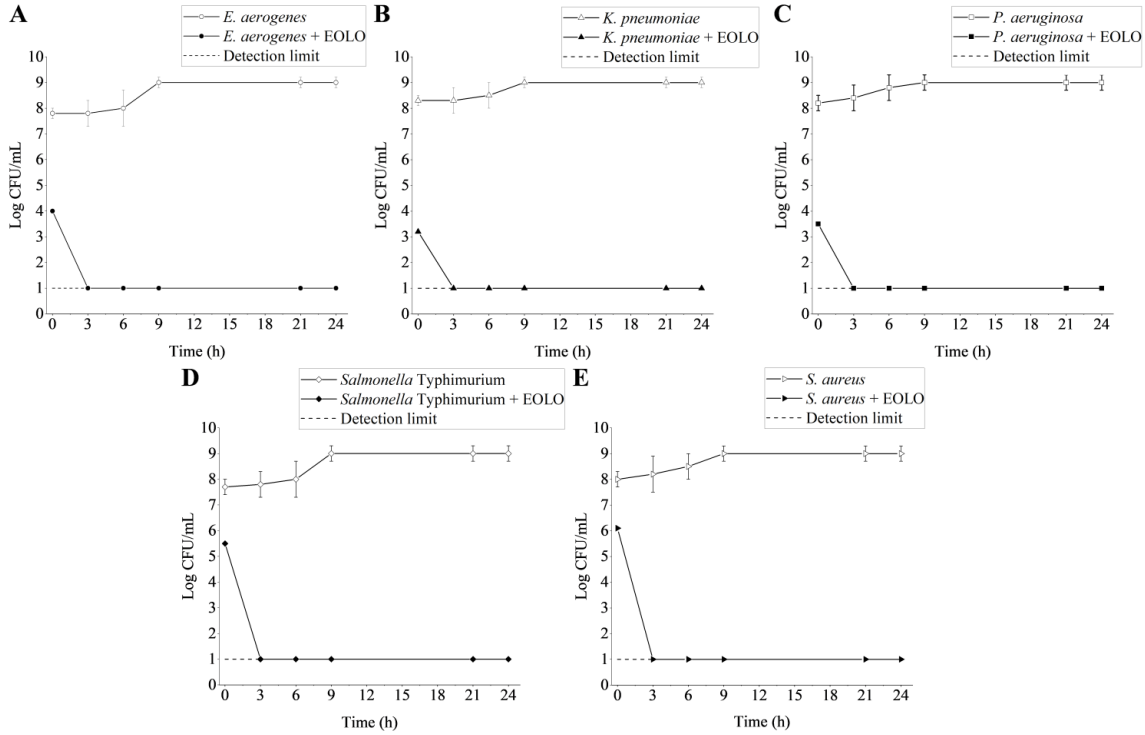


Figure 3. Bactericidal effect of the essential oil of *Lippia origanoides* Kunth (EOLO) at 10 µL/mL against *E. aerogenes* (A), *K. pneumoniae* (B), *P. aeruginosa* (C), *Salmonella* Typhimurium (D), and *S. aureus* (E). The assay was conducted for 24 h. Data were expressed as mean ± standard deviation; The dashed lines indicate the detection limit of plated by spread plate method on Mueller-Hinton agar (MH agar).

DISCUSSION

It is very important to quantify the yield of EO extraction from plant materials since they play a vital role in the economic value of the product. The yield of EOLO found by us falls within the range described in the previous studies, which varies from 0.68 to 8.00%. Cavalcanti and coauthors [8] reported EO yields ranging from 4.86 to 8.00% (w/w) from *L. origanoides* (synonym *L. sidoides*) collected from different cities in Northeast Brazil. On the other hand, Morais and coauthors [46], Morais and coauthors [47], and Mota and coauthors [48] have yields of 0.80, 0.80 and 0.68% (w/w) for the EO extracted from *L. origanoides* native to the states of Goiás, Minas Gerais, and Ceará, Brazil, respectively.

Earlier studies have identified thymol as the main metabolite of EO extracted from *L. origanoides* from different regions of Brazil [10,14-17,19,21,22,24-26,28-32,35,37,38,40-42,45,49,50,52-55,57,59,61,66-75]. However, some other studies showed that carvacrol was also the main metabolite [8,9,13,27,33,34,36,58,63,64], as well as some EOLO showed thymol and carvacrol and others only one of these two compounds. It is noteworthy that these compounds are isomers, differing structurally only by the position of the hydroxyl group of the aromatic ring, and both have antibacterial activity [76]. In contrast, Morais and coauthors [46] and Morais and coauthors [47] did not identify thymol or carvacrol as the main metabolites, but found 1,8-cineole and isoborneol as the main metabolites in the EOLO, respectively. Mota and coauthors [48] also identified caryophyllene as the main metabolite along with thymol.

In addition to the variation in the composition of the EOLO, there is also modification in the quantity of each compound. These variations, together with the EOLO extraction yield may be due to several factors, such as the location of the plant, collection period of the plant, plant age and growth stage, part of the plant dried or not, extraction method, compound identification, and quantification technique. In addition, genetic variations also play an important factor [47,77,79,80,91-97]. Factors related to plant cultivation, such as seasonality, pluviometric rate, circadian rhythm, altitude, temperature, vegetative cycles, type of soil, humidity, light intensity, the supply of water, minerals, and carbon dioxide (CO_2) can also interfere with enzymatic activities in the plant and, consequently, change the biosynthesis of some secondary metabolites, including terpenes [91,93,95,97].

Researchers reported the antibacterial potential of EOLO against several bacterial pathogens and demonstrated the presence of main metabolites possibly responsible for this property (Table 3).

Table 3. Antibacterial activity of different essential oil of *Lippia origanoides* Kunth (EOLO) described in the literature.

Bacteria	Inhibition zone diameter (mm)				MIC	MBC	MID	Main metabolite	Reference
	10 µL	20 µL	10 µL/mL	Undefined					
Gram-negative									
Aeromonas hydrophila ATCC 7966	-	-	-	-	1.25 mg/mL	1.25 mg/mL	-	thymol	Majolo et al. [40]
Aeromonas hydrophila ATCC 7966	-	-	-	-	1.25 mg/mL	1.25 mg/mL	-	-	Majolo et al. [39]
Enterobacter aerogenes ATCC 13048	-	-	-	-	2.0 mg/mL	-	-	isoborneol	Morais et al. [47]
***Enterobacter aerogenes* ATCC 13048**	**-**	**-**	**11.7**	**-**	**-**	**< 10.0 µL/mL**	**-**	thymol	**This study**
Enterobacter cloacae	-	-	-	-	0.256 mg/mL	-	-	thymol	Veras et al. [74]
Enterobacter cloacae HMA/FT502	-	-	-	-	2.0 mg/mL	-	-	isoborneol	Morais et al. [47]
Escherichia coli	13.5	23.0	-	-	< 50.0 µL/mL	-	-	thymol	Bertini et al. [17]
Escherichia coli	-	-	-	-	0.00125 mg/mL	-	-	carvacrol	Pereira et al. [58]
Escherichia coli	-	-	-	-	0.512 mg/mL	-	-	thymol	Veras et al. [74]
Escherichia coli ATCC 11229	-	-	-	-	2.0 mg/mL	-	-	isoborneol	Morais et al. [47]
Escherichia coli ATCC 25753	-	-	-	-	< 3.0 µL/mL	-	-	-	Castro et al. [23]
Escherichia coli ATCC 25922	-	-	-	5.0	< 0.015 mg/mL	-	-	thymol	Linden et al. [38]
Pool of 30 samples of *Escherichia coli* isolated of homemade Minas cheese	-	23.0	-	-	13.0 µL/mL	25.0 µL/mL	-	-	Castro et al. [23]
Klebsiella pneumoniae	-	-	-	-	0.256 mg/mL	-	-	thymol	Veras et al. [74]
***Klebsiella pneumoniae* ATCC 10031**	**-**	**-**	**10.6**	**-**	**-**	**< 10.0 µL/mL**	**-**	thymol	**This study**
Providencia rettgeri	-	-	-	-	0.256 mg/mL	-	-	thymol	Veras et al. [74]
Pseudomonas aeruginosa	0.0	0.0	-	-	-	-	-	thymol	Bertini et al. [17]
Pseudomonas aeruginosa	-	-	-	-	1.25 mg/mL	1.25 mg/mL	-	-	Melo et al. [43]
Pseudomonas aeruginosa	-	-	-	-	0.00037 mg/mL	-	-	carvacrol	Pereira et al. [58]
Pseudomonas aeruginosa	-	-	-	-	0.512 mg/mL	-	-	thymol	Veras et al. [74]
***Pseudomonas aeruginosa* ATCC 25619**	**-**	**-**	**10.3**	**-**	**-**	**< 10.0 µL/mL**	**-**	thymol	**This study**
Pseudomonas aeruginosa ATCC 9027	-	-	-	-	2.0 mg/mL	-	-	isoborneol	Morais et al. [47]
Pseudomonas aeruginosa ATCC15442	-	-	-	-	-	-	< 0.001 mg/mL air	thymol	Veras et al. [73]
Pseudomonas aeruginosa SPM1	-	-	-	-	1.0 mg/mL	-	-	isoborneol	Morais et al. [47]
Pseudomonas syringae pv.	4.7	5.9	-	-	-	-	-	carvacrol	Guimarães et al. [34]
Salmonella enterica serovar Enteritidis ATCC 13076	34.7	-	-	-	-	-	-	-	Ambrosio et al. [12]
Salmonella enterica serovar Typhi ATCC 19430	-	-	-	-	1.0 mg/mL	-	-	isoborneol	Morais et al. [47]
***Salmonella enterica* serovar Typhimurium ATCC 14028**	**-**	**-**	**11.8**	**-**	**-**	**< 10.0 µL/mL**	**-**	thymol	**This study**
Serratia marcenscens ATCC 14756	-	-	-	-	1.0 mg/mL	-	-	isoborneol	Morais et al. [47]
Xanthomonas campestris pv. *vesicatoria*	3.9	4.9	-	-	-	-	-	carvacrol	Guimarães et al. [34]

Cont. Table 3

Gram-positive									
Bacillus cereus ATCC 14579	-	-	-	-	1.0 mg/mL	-	-	isoborneol	Morais et al. [47]
Bacillus subtilis ATCC 6633	-	-	-	-	1.0 mg/mL	-	-	isoborneol	Morais et al. [47]
Clavibacter michiganensis subsp. *Michiganensis*	3.0	5.6	-	-	-	-	-	carvacrol	Guimarães et al. [34]
Enterococcus faecalis	-	-	-	-	0.512 mg/mL	-	-	thymol	Veras et al [74]
Lactobacillus plantarum ATCC 8014	37.8	-	-	-	-	-	-	-	Ambrosio et al. [12]
Listeria monocytogenes L2	-	-	-	-	-	1.0 µL/mL	-	thymol	Vázquez-Sánchez et al. [70]
Listeria monocytogenes L8	-	-	-	-	-	5.0 µL/mL	-	thymol	Vázquez-Sánchez et al. [70]
Micrococcus luteus ATCC 9341	-	-	-	-	1.0 mg/mL	-	-	isoborneol	Morais et al. [47]
Micrococcus roseus ATCC 1740	-	-	-	-	1.0 mg/mL	-	-	isoborneol	Morais et al. [47]
Staphylococcus aureus	28.0	33.5	-	-	< 50.0 µL/mL	-	-	thymol	Bertini et al. [17]
Staphylococcus aureus	-	-	-	-	0.039 mg/mL	0.039 mg/mL	-	-	Melo et al. [43]
Staphylococcus aureus	-	-	-	-	0.00063 mg/mL	-	-	carvacrol	Pereira et al. [58]
Staphylococcus aureus	-	-	-	-	0.128 mg/mL	-	-	thymol	Veras et al. [74]
Staphylococcus aureus ATCC 15656	-	-	-	20.0	< 0.015 mg/mL	-	-	thymol	Linden et al. [38]
Staphylococcus aureus ATCC 25923	-	74.4	-	-	-	-	-	thymol	Costa et al. [24]
Staphylococcus aureus ATCC 25923	-	-	-	-	0.5 mg/mL	-	-	isoborneol	Morais et al. [47]
***Staphylococcus aureus* ATCC 25923**	**-**	**-**	**10.2**	**-**	**-**	**< 10.0 µL/mL**	**-**	thymol	**This study**
Staphylococcus aureus ATCC 29213	-	-	-	-	< 3.0 µL/mL	-	-	-	Castro et al. [23]
Staphylococcus aureus ATCC 6538	-	-	-	-	0.5 mg/mL	-	-	isoborneol	Morais et al. [47]
Staphylococcus aureus ATCC12624	-	-	-	-	-	-	0.000125 mg/mL	thymol	Veras et al. [73]
Staphylococcus aureus BH1	-	18.0	-	-	0.4 µL/mL	-	-	-	Oliveira et al. [51]
Staphylococcus aureus BH10	-	18.0	-	-	0.4 µL/mL	-	-	-	Oliveira et al. [51]
Staphylococcus aureus BH13	-	15.0	-	-	0.4 µL/mL	-	-	-	Oliveira et al. [51]
Staphylococcus aureus BH14	-	18.0	-	-	0.4 µL/mL	-	-	-	Oliveira et al. [51]
Staphylococcus aureus BH15	-	15.0	-	-	0.4 µL/mL	-	-	-	Oliveira et al. [51]
Staphylococcus aureus BH17	-	18.0	-	-	0.4 µL/mL	-	-	-	Oliveira et al. [51]
Staphylococcus aureus BH18	-	16.0	-	-	0.4 µL/mL	-	-	-	Oliveira et al. [51]
Staphylococcus aureus BH19	-	21.0	-	-	0.4 µL/mL	-	-	-	Oliveira et al. [51]
Staphylococcus aureus BH2	-	18.0	-	-	0.4 µL/mL	-	-	-	Oliveira et al. [51]
Staphylococcus aureus BH20	-	18.0	-	-	0.4 µL/mL	-	-	-	Oliveira et al. [51]
Staphylococcus aureus BH5	-	18.0	-	-	0.4 µL/mL	-	-	-	Oliveira et al. [51]

Cont. Table 3

Staphylococcus aureus BH8	-	15.0	-	-	0.4 µL/mL	-	-	-	Oliveira et al. [51]
Staphylococcus aureus S8	-	-	-	-	-	0.25 µL/mL	-	thymol	Vázquez-Sánchez et al. [71]
Staphylococcus aureus S10	-	-	-	-	-	0.5 µL/mL	-	thymol	Vázquez-Sánchez et al. [71]
Staphylococcus aureus S102	-	29.0 - 60.4	-	-	-	-	-	thymol	Costa et al. [24]
Staphylococcus aureus S14	-	29.0 - 60.4	-	-	-	-	-	thymol	Costa et al. [24]
Staphylococcus aureus S16	-	29.0 - 60.4	-	-	-	-	-	thymol	Costa et al. [24]
Staphylococcus aureus S26	-	29.0 - 60.4	-	-	-	-	-	thymol	Costa et al. [24]
Staphylococcus aureus S27	-	29.0 - 60.4	-	-	-	-	-	thymol	Costa et al. [24]
Staphylococcus aureus S46	-	29.0 - 60.4	-	-	-	-	-	thymol	Costa et al. [24]
Staphylococcus aureus S81	-	29.0 - 60.4	-	-	-	-	-	thymol	Costa et al. [24]
Staphylococcus aureus S82	-	29.0 - 60.4	-	-	-	-	-	thymol	Costa et al. [24]
Staphylococcus aureus S83	-	29.0 - 60.4	-	-	-	-	-	thymol	Costa et al. [24]
Staphylococcus aureus S86	-	29.0 - 60.4	-	-	-	-	-	thymol	Costa et al. [24]
Pool of 30 samples of *Staphylococcus aureus* isolated of homemade Minas cheese	-	26.0	-	-	13.0 µL/mL	25.0 µL/mL	-	-	Castro et al. [23]
Staphylococcus epidermidis	-	-	-	-	0.00063 mg/mL	-	-	carvacrol	Pereira et al. [58]
Staphylococcus epidermidis ATCC 12229	-	-	-	-	1.0 mg/mL	-	-	isoborneol	Morais et al. [47]
Streptococcus agalactiae KJ561060	-	-	-	-	0.3125 mg/mL	0.4167 mg/mL	-	thymol	Majolo et al. [41]
Streptococcus mitis	10.0	-	-	-	10.0 mg/mL	40.0 mg/mL	-	thymol	Botelho et al. [19]
Streptococcus mutans	-	-	-	-	0.256 mg/mL	-	-	thymol	Veras et al. [74]
Streptococcus mutans ss-980	18.7	-	-	-	5.0 mg/mL	20.00 mg/mL	-	thymol	Botelho et al. [19]
Streptococcus salivarius	8.5	-	-	-	10.0 mg/mL	40.0 mg/mL	-	thymol	Botelho et al. [19]
Streptococcus sanguis	12.0	-	-	-	10.0 mg/mL	40.0 mg/mL	-	thymol	Botelho et al. [19]

Minimal inhibitory concentration (MIC); Minimum bactericidal concentration (MBC); Minimal inhibitory dose (MID); - = not identified or not informed or unperformed; In bold the result of this study.

Similarly, we have explored the antibacterial potential of EOLO and revealed the presence of different metabolites in this study. Therefore, we associate the effect of EOLO against bacteria with the presence of the main metabolites (Table 1). Interestingly, thymol and carvacrol are isomers and the main metabolites found in the EOLO, which may be responsible for the antibacterial activity of EOLO against Gram-negative and Gram-positive bacterial pathogens [76]. Generally, the antibacterial activity of EO is dependent on the mechanism of action of each compound and the synergistic, additive, or antagonistic interactions between them. Therefore, the variable composition of EO makes it difficult to compare its antibacterial activities between studies [76,78,79]. On the other hand, this mixture of compounds with different mechanisms of action can significantly help in the multidrug-resistant bacteria control, serving as a scaffold to the development of synthetic drugs, reducing the risk of emergence of new resistant bacteria [73,76,80].

In addition to variations in the composition of the EOLO making it difficult to compare the results of antibacterial activity, the bacterial strains, microbiological techniques, and concentrations of the EOLO are also acting as the limiting factors, as shown in Table 3. Van de Vel and coauthors [79] showed that different incubation conditions, culture media, and the use of emulsifiers/solvents significantly influence the MIC of EO. These authors also pointed out that there is a need for a good international standard procedure to assess the antibacterial activity of EO for comparative studies. In general, despite all the difficulties in comparing the antibacterial effects by the EOLO, these EO have efficient growth inhibitory activity against both Gram-negative and Gram-positive bacterial pathogens even at low concentrations (Table 3).

CONCLUSION

The EOLO showed strong antibacterial activity against Gram-negative and Gram-positive bacterial pathogens at low concentrations. Thus, the use of EOLO as an antibacterial agent in the food and pharmaceutical industries is promising.

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